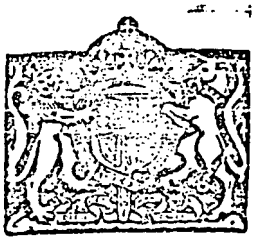
PATENT SPECIFICATION



Application Date: Dec. 7, 1942. No. 17365/42. 568,384

Complete Specification Accepted: April 3, 1945.

COMPLETE SPECIFICATION

Whooping Cough Toxin

I, HAROLD EDWIN POTTS, Chartered Patent Agent, of 12, Church Street, Liverpool, in the County of Lancaster, Subject of the King of Great Britain, do hereby declare the nature of this invention which has been communicated to me by Ayerst-McKenna & Harrison Limited, a Corporation of the Dominion of Canada, of 485, McGill Street, in the City of Montreal, Province of Quebec, Dominion of Canada, and in what manner the same is to be performed, to be particularly described and ascertained in and by the following statement:—

This invention relates to a useful biological product having the characteristics of a toxin specific to whooping cough (hæmophilus pertussis) to its preparation, and to allied substances and their preparation.

Whooping cough is today recognized by public health authorities as causing a greater proportion of infant fatalities, in America, than any other disease. This is probably due to the fact that there has been a lack of progress in specific treatment and in detecting susceptibility for whooping cough, whereas efficient methods have been made available for most infectious diseases. While there are fairly satisfactory methods capable of immunizing a large proportion of children susceptible to whooping cough, the mortality rate of this disease is highest at the fifth month, and immunization of infants under six months of age is fraught with considerable danger. Such young children are liable to possess in the bloodstream antibodies received from the mother at the time of birth, so that an injection of bacterial vaccine containing hæmophilus pertussis may react with the immune serum in bringing about anaphylactic shock or other dangerous reactions, not infrequently with fatal effects.

It is thus most desirable to know, in advance, the immunological state of infants so that those already immune and likely to react dangerously will not be inoculated. It is also desirable, in the event of epidemics, to know what children are immune so that they will not have to be quarantined.

To determine immunity to other diseases, substances have been developed, which can be applied in simple skin tests. While investigation of pertussis has gone on for a considerable period, there is no indication that there has been offered a product suitable for use as a practical skin test specific to this disease. The contributions to the field have been principally in the nature of bacterial vaccines for active immunity which have, for their purpose, produced fairly reliable and successful results, but they are not applicable to skin tests.

It has been proposed, however in specification No. 433,910 to produce whooping cough antigens from hæmophilus bacilli by bursting the structure of the bodies of the bacteria by for example repeated freezing and thawing, followed by centrifuging the suspension.

OBJECTS

Having regard to the foregoing, it is one of the principal objects of the present invention to provide a toxic substance specific to whooping cough, which can be used in a skin test for determining the immunological state of a subject. Another important object of the invention is to provide substances of an allied nature, which may be used for active and passive immunity. Another object is to provide convenient and practical processes for producing these substances.

PREPARATION OF THE PRODUCT

Considerable experimentation, on the part of my foreign correspondents led to the belief that there was present in hæmophilus pertussis a substance having the characteristics of an endotoxin specific to the disease and that serologically this endotoxin had certain useful applications, among which was its aptness for use in a practical skin test on human beings for the determination of their immunological state. Owing to the nature of hæmophilus pertussis, difficulty was experienced, at first, in obtaining a substance satisfying the necessary criteria and sufficiently stable for practical

application.

Eventually there were evolved methods of obtaining a product having the desired qualities. In one method, H. pertussis, phase 1, was cultured on a suitable medium. After incubation, the culture was suspended in physiological saline and the suspension frozen and thawed a number of times. The suspenion was then dried, *in vacuo*, to concentrate the electrolyte after which this dried material was resuspended in distilled water, allowed to stand for a period, and then centrifuged. The supernatant liquid thus obtained contained an active water soluble extract having the characteristics of an endotoxin specific to pertussis.

The purpose of concentrating the electrolyte is primarily to increase the osmotic pressure so that when water is subsequently added the membrane is ruptured by virtue of the increase of osmotic pressure.

To exemplify the reference to hæmophilus pertussis phase, I, it should be mentioned that hæmophilus pertussis after isolation from the human subject may pass through various phases as described by Leslie and Gardner (J. Hygiene 31: 423 (1931). These phases are designated I, II, III, and IV of which phase I is the smooth, virulent phase as is found in recently isolated cultures; phase IV is the rough avirulent phase; and phase II and III are considered as intermediates.

In another method, which has been found even more efficient, the culture is first washed and is then suspended in hypertonic (as compared with physiological) saline. It is then subjected to the freezing and thawing steps.

Use in a Skin Test

This active extract, in high dilution, is lethal for mice, produces dermonecrosis in the skin of rabbits or guinea pigs. Also in high dilution, it produces an erythematous patch in children not immune to pertussis, without the slightest allergic reaction. Moreover, the substance is quite stable, retaining its toxic effect for months when kept at 4.0° C.

Sodium ethylmercuri-thiosalicylate 1 in 10,000 has been found to be a satisfactory preservative (to prevent bacterial growth) for the toxin, in that it does not destroy its toxicity.

Examples

Now that the nature of the invention has been outlined and in order that it may be understood in a more specific sense, actual examples will be given, as follows, of actual procedures which are specific embodiments of the invention. It will be understood that the data given in these examples is furnished only for illustrative purposes and is not to be taken in a limiting sense.

Example 1.

"Bordet-Gengou" medium, modified by the addition of 1.5% dialyzed "Difco" proteose peptone, was poured into six small Petri dishes, with each dish measuring approximately 9.6 square inches area × 0.25 inches thickness. The medium of all plates was streaked with the freshly-isolated bacteria, which satisfied biologically and serologically the criteria of hæmophilus pertussis phase 1. The plates were incubated aerobically for 72 hours at 37° C. and confluent growth thus obtained. The culture of the plates, estimated to include on an average 50×10^{10} bacteria per plate, was then scraped, washed in saline and suspended in 30 c.c. hypertonic saline in a centrifuge bottle. The suspension was frozen in carbon dioxide snow and ethylene glycol monomethyl ether (−70° C.) and then allowed to thaw in a water bath at 37° C., the freezing and thawing being repeated ten times. The thawed suspension was then dried for about 8 hours, *in vacuo*, over phosphorus pentoxide (P_2O_5), leaving a dried mass of a brownish colour adhering to the bottle. After that time, the dried mass was resuspended in 30 c.c. sterile distilled water, shaken for 30 minute and then kept in cold (at 4.0° C.) for 24 hours. The material was centrifuged at high speed and the supernatant liquid separated. The supernatant liquid thus obtained was of a pale yellowish colour and was found to contain a water soluble extract having the characteristics of an endotoxin specific to pertussis, as set forth in the following Examples 3, 4 and 7. Precipitation of the endotoxin was effected using 2% acetic acid, precipitating the endotoxin at a p_H of 7, at which point it goes into solution readily. These endotoxin solutions form precipitates with excess ethyl alcohol, with 1% sulphosalicylic acid and with 5% trichloracetic acid. No coloration was produced with dilute iodine solution.

Micro-nitrogen determination (Kjeldahl) for three different preparations of the endotoxin solution gave the following figures:—0.203—0.142 and 0.162 mg. The endotoxin solution also contained 0.06 mg. of hydrolysable phosphorus per cc.

The purified endotoxin (15.6 m.g.) from 130 cc. of the crude endotoxin preparation was redissolved in water at p_H 7.0 to give a clear slightly yellow coloured solution. This solution gave a white precipitate with 5% trichloracetic acid or with an excess of alcohol, and positive

Biuret, Millon's, Hopkins-Cole and xanthoproteic tests were obtained.

These results indicate the protein characteristics of the precipitate and the presence of tyrosine and of tryptophane.

Glycuronic acid (0.6%) was present in acid hydrolysates of purified toxin preparations (boiling 3.5 N. HCL for 3½ hours), as determined by the method of Maughan, Evelyn and Browne—Journal of Biological Chemistry, 126: 567 (1938).

The sugar content (calculated as glucose) was 3.4% of which approximately half was present as "fermentable sugar".

EXAMPLE 2.

A procedure was carried out similar to that of Example 1 with the exception of the freezing and thawing step. In this instance, the suspension was frozen slowly in ice and salt and then allowed to thaw in a water bath at 37° C. The freezing and thawing was repeated three times. The suspension was then treated as in Example 1, with the result that a supernatant liquid was obtained containing an active water soluble extract having the characteristics of an endotoxin specific to hæmophilus pertussis, determined as in the following Examples 3, 4 and 7. The supernatant liquid was of a pale yellowish colour, was not dialyzable, nor filtrable through a Seitz filter without a great loss in potency, but filtrable through a Jena 3 sintered glass filter.

The dried mass obtained from the drying step in Example 2 was suspended in 30 c.c. distilled water, kept in the cold (4° C.) for 24 hours and then centrifuged and the supernatant liquid separated. 1.0 c.c. of the supernatant liquid thus obtained, injected intraperitoneally killed white mice weighing 10 to 22 gms. within 18 hours. Before death, the mice became very bluish in colour, as if cyanosed. This was particularly visible in the mouse's tail.

Serial dilutions were made of the supernatant liquid containing the endotoxin and the mice were injected intraperitoneally each with 1.0 c.c. of the particular dilution tested. The results as expressed in the following Chart 1.

CHART 1

Dil of Endotoxin	Wt. in Mg./cc.	24 hours		48 hours	
		D	S	D	S
Undiluted	11.130	10	0	10	0
1/2	5.565	10	0	10	0
1/4	2.783	10	0	10	0
1/8	1.392	10	0	10	0
1/16	0.696	7	3	9	1
1/32	0.348	0	10	0	10
M.L.D. 1/10	1.113				

In chart 1 the first column expresses the dilution of supernatant liquid, the second the weight in mg. per c.c. of the dried total solids containing endotoxin present in the dilution. The third and fourth columns express the lethal effect of the respective doses on mice, D indicating "died" and S indicating "survived".

From the information given in this chart, it is found that when the dried mass containing the endotoxin was diluted 1:8, representing 1.392 mg. of endotoxin, all of the mice (10) died in 24 hours. When half of this amount i.e. 1/16 solution was given 9 of 10 mice died in 48 hours while none of 10 mice died when the solution was diluted 32 times. Thus the minimal lethal dose for mice as established by experiment could be considered as 1.113 mg. of endotoxin or a dilution of 1:10. The equivalent of this dose contained in 0.2 cc., when injected intradermally into rabbits or guinea pigs, produced inflammation purplish in colour within 8 hours, which was followed by necrosis after 96 hours. Dried organisms obtained after the drying step of Example 1 treated in exactly the same manner were found to produce substantially identical reactions.

EXAMPLE 4.

The dried material taken after the drying step of Example 1 was suspended in 600 c.c. distilled water, kept in the cold for 24 hours, centrifuged, and the supernatant liquid separated. It was found that 0.1 c.c. of the supernatant liquid thus obtained injected intradermally into children whose histories were essentially negative to whooping cough, into children who had lost their immunity to the disease, produced a skin reaction in the form of an erythematous area

averaging 1 to 3 cm. in diameter within 6 to 24 hours, which faded about 12 hours afterwards. Infants and children with case histories substantially positive to whooping cough reacted negatively to the test. The following Chart 2 indicates the precise result obtained from a large clinical trial on the action of the toxin of this example in skin tests. The dried material taken after the drying step of Example 2 treated in exactly the same manner was found to produce similar reactions.

EXAMPLE 4A.

In another example a procedure was carried out similar to that of Example 1, except that the harvested organisms were washed 3 times in saline before being resuspended in hypertonic saline, and then frozen and thawed 10 times.

It was found in another clinical trial that when 0.1 cc. of the endotoxin containing 1/20 M.L.D. for mice was injected intracutaneously into children a skin reaction was produced which measured about 1 to 2 cm. in diameter and which was blanched. This occurred in the first 48 hours. The blanched area became erythematous and the red colour became deeper and the height of the reaction was observed on the 5th day, after which time the colur faded into a light brown which persisted for several months. Those negative to the test showed only a blanched area which disappeared on the 5th day.

CHART 2

Number of children assayed: 100
Dose: 0.1 cc. dilution 1/600

	All children assayed	Infants
Incidence of whooping cough	17%	0%
Toxin negative	19%	30%
Toxin positive	81%	70%

	Children 8 and over
Incidence of whooping cough	21%
Toxin negative	16%
Toxin positive	84%

	Children 8 and over	Under 8 yrs.
History of whooping cough	15	2
Toxin positive	10	1
Toxin negative	5	1

	6 Hrs	12 Hrs.	24 Hrs.	Between 6—24 Hrs.
Positive	42	37	56	81
Negative	58	43	44	19

Retest on Negatives and Doubtfuls

Number of children assayed:	38	
Dose: 0.1 c.c. dilution 1/300		
Toxin positive	36	
Toxin negative	2	

Retest on Positives

Number of children assayed:	37	
Dose: 1.1 c.c. dilution 1/1200		
Toxin positive	28	75%
Toxin negative	9	25%

EXAMPLE 5.

30 cc. of the substance (the supernatant liquid of Example 1) was kept in a flask at 4.0° C. and was tested as to biological activity once each month for four months. In each test the activity was maintained, in that the liquid was diluted 10 times and 1.0 cc. injected intraperitoneally

killed white mice in 18 hours and 0.2 cc. produced characteristic necrotic lesions in the skin of a rabbit, 1/20 minimal lethal dose for mice, injected intradermally, produced an erythematous patch in susceptible children. 30 c.c. of the supernatant liquid of Example 2 was similarly aged and tested with substantially identical results.

EXAMPLE 6.

10 rabbits of the chinchilla or white type, each weighing 5 to 7 pounds, were injected subcutaneously by a series of increasing doses over a period of time, with pertussis endotoxin produced by the method of Example 1 and formolized with 0.4% formalin. The treated rabbits were given a rest period and then bled from the heart; the serum separated from the clotted blood contained the anti-endotoxin. Test tubes containing 20,000 million pertussis Phase I organisms as a suspension in 1.0 cc. of saline failed to agglutinate in the presence of 1 c.c. of serum in dilutions greater than 1/100. when incubated at 37° C. for 1 hour and overnight at 4° C. Normal rabbit serum frequently agglutinates H. pertussis phase I in a titre of 1/100. On the other hand this suspension agglutinated with serum obtained from another group of rabbits injected with hæmophilus pertussis phase I organisms in dilution of the serum up to 1/10,000.

In order to show that the pertussis anti-endotoxic serum was of high value and was specific for the endotoxin the following experiments were performed. Two groups of mice were tested with serum culture mixture, one group received live hæmophilus pertussis phase I organisms and hæmophilus pertussis phase I antibacterial serum; the other group received hæmophilus pertussis phase I organisms and pertussis anti-endotoxin.

CHART 3

TOXIN NEUTRALIZING EFFECT OF PERTUSSIS ANTI-ENDOTOXIN SERUM
(0.5 cc. endotoxin solution (2 M.L.D.) +0.5 cc. diluted anti-endotoxin)*

Anti-endotoxin I		Anti-endotoxin II	
Dilution	Deaths in 10 days	Dilution	Deaths in 10 days
Undiluted	0/10	Undiluted	0/10
1/2	0/10	1/2	0/10
1/4	0/10	1/4	0/10
1/8	10/10	1/8	0/10
1/16	10/10	1/16	0/10
1/32	10/10	1/32	0/10
1/64	10/10	1/64	10/10
Toxin only	10/10 (36 hrs.)	Toxin only	10/10 (36 hrs.)
Anti-endotoxin only	0/10	Anti-endotoxin only	0/10

*Endotoxin-anti-endotoxin mixture kept at room temperature for 3 hours prior to the intraperitoneal injection of the mice

Anti-endotoxins I and II were merely two different batches of the same material, but on titration of potency the latter had more than 4 times the potency of the former. This is a common finding in biological assays, even of the same material.

Chart 3 is designed to show the endotoxin neutralizing effect of pertussis anti-endotoxin. When 2 M.L.D. of endotoxin contained in 0.5 c.c. mixed with 0.5 c.c. of the anti-endotoxin in halving dilutions were kept at room temperature for 3 hours and 1 c.c. of this mixture injected intraperitoneally into mice, it was found that the anti-endotoxin could be diluted 32 times and still retain neutralizing value against 2 M.L.D. of endotoxin but failed to completely neutralize in a dilution of 1:64. The mice were observed for 10 days. In the controls where 2 M.L.D. of endotoxin was given without protective anti-endotoxin, the mice died in 36 hours.

PROPERTIES OF THE PRODUCT.

From the results of skin tests on human beings similar to those demonstrated by Example 4, it was found that 1/20 of a minimal lethal dose for mice, as determined by the tests, served as a suitable standard for human use. Anything appreciably over this amount produced positive reactions in children whose histories were substantially positive towards pertussis, whereas anything substantially under this amount, might produce negative reactions in children with histories substantially negative to pertussis. The

great utility in this standardized form of the product, for use in skin tests, can readily be seen, in that its results are highly accurate. Serial dilution of the endotoxin as indicated by Chart 1 in conjunction with the clinical trial as indicated by Chart 2 enables the determination of the degree of susceptibility of a child to whooping cough. The product may be used quite safely in prescribed concentration.

Observations on children on which the skin test was performed, as for instance according to Example 4, indicated that there was no bacterial antigen reaction. This is believed to be due to the purity of the product, that is to say its freedom from bacteria or fragments thereof. This is made evident from tests, such as those of Example 6, where a suspension of H. pertussis organisms phase I failed to agglutinate with the anti-endotoxin at a titre higher than normal rabbit serum, whereas the same suspension agglutinated with a serum from rabbits immunized with the organisms at a titre of 1/16,000.

The stability of the endotoxin, demonstrated for instance in Example 5, makes possible the diagnostic use of the product on a large scale. It can be kept for long periods without unusual precautions.

PREPARATION.

Regarding the preparation of the pertussis endotoxin, it is believed that the steps of repeated freezing and thawing, in combination with drying to concentrate the electrolyte and subsequent resuspension in water, offer a particularly advantageous method for the isolation of the substance in the pure condition such that it has desired specific toxicity and wherein it may be used in a human skin test without undesirable reactions. My foreign correspondent's explanation of the success of this method, as opposed to others which were attempted, is that haemophilus pertussis organisms are extremely resistant to deleterious agents and drastic treatment requiring the first step of repeated freezing and thawing to weaken the more resistant organisms and to kill the weaker ones, and the increased osmotic pressure produced by the resuspension in water after drying to so modify the permeability of the membranes as to release an endotoxin.

IMMUNIZATION.

It has also been found that the endotoxin prepared according to the invention may be detoxified with formalin to form an endotoxoid and injected into animals either subcutaneously or intranasally or by any other route known to produce the desired response to produce a serum capable of neutralizing the effect of the endotoxin (Example 6 and Chart 3).

The use of the toxin for the preparation of antitoxin is further demonstrated by the following example.

EXAMPLE 7.

An endotoxin obtained according to Example 1 was formolized with 0.4% formalin. The endotoxoid thus produced was injected subcutaneously into rabbits and subcutaneously into mice and found to be antigenic and to produce serum in these animals which would neutralize the effect of the endotoxin. Thus it was established that the endotoxoid can be used for active immunity against haemophilus pertussis and the rabbit serum for passive immunity. A comparison of active immunization with haemophilus pertussis phase I organisms and pertussis endotoxoid is found on the following Chart 4.

CHART 4

Immunized with	Tested with
Group A Killed H. pertussis	2 M.L.D. Live H. Pertussis
Group B Killed H. pertussis	2 M.L.D Pertussis Endotoxin
Group C Pertussis Endotoxoid	2 M.L.D. Live H. Pertussis
Group D Pertussis Endotoxoid	2 M.L.D Pertussis Endotoxin

Days	1st		2nd		3rd		4th		5th		6th		7th		8th		9th		10th	
	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S	D	S
Group A	0	20	2	18	4	16	7	13	9	11	11	9	12	8	15	5	18	2	20	0
Group B	5	15	15	5	20	0														
Group C	0	20	0	20	0	20	0	20	0	20	0	20	1	19	2	18	2	18	2	18
Group D	0	20	0	20	1	19	2	18	3	17	4	16	5	15	5	15	5	15	5	15

Controls				
H. pertussis	3	2	5	0
Endotoxin	5	0		

N.B. Controls were not previously immunized.

Chart 4 shows the difference in degree of immunity produced in mice by active immunization with pertussis bacterial vaccine and pertussis endotoxoid. 4 groups of 20 mice each were given a series of injections: 2 groups receiving bacterial vaccine and the other 2 groups endotoxoid. These animals were injected later with either 2 M.L.D. of live pertussis organisms or 2 M.L.D. of endotoxin.

It was found that in the group of 20 mice immunized with pertussis bacterial vaccine none of the mice survived at the end of ten days when injected with 2 M.L.D. of living organisms, whereas a similar group immunized with the same vaccine were all dead at the end of 48 hours when injected with 2 M.L.D. of endotoxin. But in the group of mice immunized with pertussis endotoxoid 18 of 20 survived at the end of 10 days when injected with 2 M.L.D. of live pertussis organisms while in the 4th group, also immunized with endotoxoid, 15 of 20 mice survived at the end of 10 days when injected with 2 M.L.D. of endo-toxin. The controls, which were not immunized previously, all died in 48 hours when given 2 M.L.D. of live H. pertussis organisms and in a similar group treated with 2 M.L.D. of endotoxin all died in 24 hours.

Haemophilus pertussis phase I organisms have been specified in the Examples. This phase is known to be the most potent, but naturally other phases might be used with varying results.

It will also be understood that the above disclosure is intended in an illustrative sense and that various modifications may be made in the procedure outlined, to achieve results within the purview of the invention. It is intended that the scope of the patent be limited only by the state of the prior art and the scope of the following claims.

Having now particularly described and ascertained the nature of the said invention and in what manner the same is to be performed, as communicated to me by my foreign correspondents, I declare that what I claim is:—

1. A process for producing a substance having the characteristics of an endotoxin specific for pertussis which includes the steps of suspending pertussis organisms in a saline solution, repeatedly freezing and thawing said suspension, drying the suspension to concentrate the electrolyte and to form a dried mass containing said endotoxin, and releasing the endotoxin from said dried mass by re-suspending said dried mass in sterile distilled water.

2. A process as claimed in claim 1 in which the soluble and insoluble fractions, of the re-suspension are separated the latter being discarded.

3. A process as claimed in claim 2 in which the resuspension is centrifuged, and the supernatant liquid containing the endotoxin is separated therefrom.

4. A process as claimed in claim 3 in which preservative is added.

5. A process as claimed in any of the preceding claims in which the organisms are haemophilus pertussis phase I.

6. A process as claimed in any of the preceding claims in which the endotoxin is de-toxified by means of formalin and converted into an endotoxoid.

7. A process of preparing an anti-endotoxin specific to pertussis which consists in preparing an endotoxoid as in claim 6, injecting an animal therewith, and separating a serum from the blood of the said animal.

8. An endotoxin specific to pertussis whenever prepared or produced by the process claimed.

9. An endotoxoid-specific to pertussis whenever prepared or produced by the process claimed.

10. An anti-endotoxin specific to pertussis whenever prepared or produced by the process claimed.

Dated this 5th day of December, 1942.
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Chartered Patent Agents.

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